SUPPLEMENTAL MATERIAL

Similarity of Bisphenol A Pharmacokinetics in Rhesus Monkeys and Mice: Relevance for Human Exposure

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Table of Contents

Part 1

A. Chemicals page 3
B. Experiment 1
   1. *Deuterated BPA (dBPA) administration and sample collection for monkeys*
   2. Isotope dilution LC-MS analysis of unconjugated and conjugated dBPA
C. Experiment 2A
D. Experiment 2C
   1. *HPLC-CoulArray analysis of unconjugated and conjugated BPA*

Part 2

A. Means, SE, and number of animals per group from each experiment (Tables 1–5) page 5
B. Pharmacokinetic and statistical analyses using WinNonlin and NONMEM software
   1. *Non-compartmental analysis (experiment 1 with rhesus monkeys and experiment 2A with mice)*
   2. *Compartmental analysis (experiment 1 in rhesus monkeys)*
   3. *Dose proportionality (experiment 2B in mice)*
C. Figures page 17

Part 1

A. CHEMICALS

Tritiated BPA (^{3}H-BPA; specific activity 7.3 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA), and unlabeled BPA (>99% pure) was obtained from Aldrich (Milwaukee, WI). Tocopherol-stripped corn oil was from MP Biomedicals (Solon, OH). Methanol, water and tert-butyl methyl ether were HPLC grade and obtained from Fisher Scientific (Pittsburgh, PA). Deuterated (d6)-BPA was purchased from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada).

Water used in these studies was tested for the presence of background BPA, after concentration on C18 Sep-paks (see Experiment 2C, below). BPA was not detected in this water, even after a 250-fold concentration. Other sources of laboratory water tested had values of 0-0.16 ng/mL. We have no evidence for BPA leaching from the HPLC equipment or solvents; blank samples did not appear to contain BPA, and spiked samples gave anticipated values.

B. EXPERIMENT 1

Deuterated BPA (dBPA) administration and sample collection for monkeys. All animals were trained to accept small pieces of fruit prior to beginning the dBPA treatment period. Fruit was small enough that animals would take the fruit in one bite and did not try to pull it into smaller pieces prior to consuming it. Preferences of each animal were noted. The dBPA dose for each animal was calculated based on body weight the day before the treatment period began. dBPA was prepared as a 25 mg/mL ethanol stock solution, and the daily dose fed was 400-µg/kg body weight given daily in the morning for 7 days. The dBPA/ethanol solution (100-150 µL) was injected with a Hamilton 200 µL syringe into the center of fruit pieces, such as grapes, banana slices, dates or dried apricots, so that the animal could grasp the fruit and place it in its mouth without touching the dBPA.

Isotope dilution LC-MS analysis of unconjugated and conjugated dBPA. Serum samples (1-2 mL) were spiked with ^{13}C-BPA (Cambridge Isotopes Laboratories, Andover, MA) as an internal standard, and extracted twice with methyl tert-butyl ether for determination of unconjugated dBPA. The ether extract was dried under nitrogen and reconstituted in 60:40 methanol:water. After extraction of unconjugated dBPA, for analysis of unextracted conjugated dBPA (glucuronidated and sulfated forms), the samples were treated overnight at 37^{o}C with b-glucuronidase/aryl sulfatase (Sigma) and then extracted by the same procedure used for unconjugated dBPA.

Serum dBPA was assayed by LC-MS using a Thermo Finnigan Surveyor MSQ plus connected to an integrated Thermo-Accela LC system; analytes were detected using electrospray ionization with negative polarity, a cone voltage of 70V, and probe temperature of 600^{o}C. Separations were performed on a 1.9 micron Hypersil Gold HPLC column (50x2.1 mm) with a mobile phase gradient running from 20% to 95% acetonitrile over 6 minutes, at 550 µL/minute. dBPA and ^{13}C-BPA were detected using selected ion monitoring for m/z 233 and m/z 239 respectively. Thermo Xcalibur software was used to autotune, acquire, and process the LC/MS data. Isotope dilution quantitation was made against a standard curve of at least 5 calibration standards (dBPA and ^{13}C-BPA) to adequately cover the expected BPA concentration range. The limit of quantitation (LOQ) for BPA in serum was 0.2 ng/mL (parts

per billion, ppb) based on extraction of 2 mL of serum, which was at least 5 times background. The coefficient of variation for the LOQ was 8%. Intra- and inter-assay coefficients of variation, derived from five assays, were 9.8% and 18.3% respectively. The standard curves were linear (for example, $R^2 = 0.9778$) based on visual inspection.

C. EXPERIMENT 2A

Methods for measuring unconjugated ^{3}H-BPA in serum. Two volumes of cold absolute methanol were added to volumes of serum ranging from 150-350 mL. Precipitated proteins were pelleted at 4^{o}C by centrifugation for 15 minutes at 3,000 x *g*. The supernatant was dried under nitrogen, and brought to 50% methanol by the addition of 75 mL methanol and 75 mL distilled deionized H_2O. The reconstituted samples were separated by HPLC on a reverse phase Hypersil C18 column (4.6 x 100 mm, Phenomenex), using a mobile phase of 65% methanol at a flow rate of 0.55 mL/min, as previously described (Taylor et al. 2008). Elution of separated components was monitored by UV absorbance at 260 nm on a Perkin-Elmer LC-90 spectrophotometric detector, and also using a bRAM in-line scintillation counter (IN/US Systems, FL) to monitor radioactivity. Authentic ^{3}H-BPA (Moravek) was used as a standard to identify expected elution times. Fractions from injected samples were collected at 20-second intervals across a window spanning the authentic BPA elution time, and radioactivity per fraction was counted on a scintillation counter for 10 minutes/sample (this provides greater sensitivity and accuracy than the bRAM measurements).

BPA was quantified by summing the radioactivity in the fractions eluting at the same time points as authentic BPA. Counts per minute (cpm) were converted to mass by referencing the specific activity of the original administered oil sample. The sensitivity of the assay, calculated as two-fold above background cpm, was 0.28 ng BPA/mL serum.

The running time for BPA was verified at regular intervals using ^{3}H-BPA and also using positive control samples, which consisted of untreated mouse serum containing ^{3}H-BPA (~2700 cpm per 100 µL). The recovery of the added ^{3}H-BPA, determined by comparing the sum of the radioactivity measured in the HPLC fractions to radioactivity in spiked plasma that had not been extracted, averaged (±SEM) 84.1±10.4% across 4 positive control sample runs. Background counts, determined individually for all sample runs were similar, averaging 13.17±0.868 cpm. Mouse sample values were adjusted for recovery.

D. EXPERIMENT 2C

HPLC-CoulArray analysis of unconjugated and conjugated BPA. Two volumes of cold absolute ethanol were added to serum. Precipitated proteins were pelleted at 4^{o}C by centrifugation for 15 minutes at 3,000 *g*. The supernatant was brought to 600 µL using High Performance Liquid Chromatography (HPLC)-grade water (Fisher Scientific) and passed through a C18 Sep-Pak SPE cartridge (Waters). Sep-pak cartridges were pre-washed with 15 mL methanol to remove potential BPA contamination; prior tests had determined that BPA leakage was variable, but that the highest levels seen ere removed by this pretreatment. The SPE eluate was dried down under nitrogen, and then reconstituted in 50% methanol for HPLC separation. Conjugated BPA (glucuronidated and sulfated forms) was determined using the same sample preparation after treatment of 100 µL aliquots of serum overnight with ß-glucuronidase/aryl sulfatase (Sigma). Concentrations of BPA in sample extracts were determined by HPLC with an ESA CoulArray 5600 detector. Separation was performed on a reverse-phase 250 mm Prodigy C18 column

(Phenomenex), with a mobile phase of 36:24:40 acetonitrile: methanol: 0.05 M sodium acetate buffer (pH 4.8), and with the CoulArray cell potentials set at 325, 400, 720 and 875 mV. The limit of detection under these conditions was 9 ng/mL. Extraction efficiency was assessed using mouse serum samples spiked with 5 ng BPA, extracted as described above; recoveries averaged 89.97%. The intra-assay coefficient of variation, based on the analysis of 12 internal standards, was 1.4%.

Part 2

A. MEANS, SE, AND NUMBER OF ANIMALS PER GROUP FROM EACH EXPERIMENT

Table 1. Experiment 1 data for unconjugated and conjugated serum dBPA in adult female rhesus monkeys over the 24 hr after a single (day 1) or seven (day 7) consecutive days of oral exposures to a 400 µg/kg dBPA. Each monkey was repeatedly bled, 8 times over 24 hr, on day 1 and day 7.

		Unconjugated dBPA, ng/mL			Conjugated dBPA, ng/mL		
	Time (hr)	Mean	SEM	*n*	Mean	SEM	*n*
Day 1	0	0.00	0.00	11	0.13	0.07	8
	0.5	3.05	0.75	11	140.02	51.68	8
	1	3.95	0.55	11	134.25	42.41	8
	2	1.96	0.30	11	149.47	14.58	8
	4	0.63	0.11	11	114.39	43.43	8
	8	0.34	0.15	11	39.96	14.24	8
	12	0.15	0.08	11	13.44	4.83	8
	24	0.08	0.05	11	10.54	7.11	8
Day 7	0	0.07	0.04	10	9.59	4.30	10
	0.5	3.15	0.42	10	104.40	19.51	10
	1	4.40	0.54	10	226.96	46.23	10
	2	1.87	0.17	10	150.68	39.67	10
	4	0.60	0.12	10	88.02	17.01	10
	8	0.18	0.05	10	38.19	8.03	10
	12	0.13	0.04	10	40.94	17.36	10
	24	0.04	0.02	10	21.93	13.35	10

Table 2. Experiment 2A data for unconjugated serum ^{3}H-BPA in adult female CD-1 mice over the 24 hr after a single oral administration of 400 µg/kg ^{3}H-BPA. Values at each time point were from different animals.

Time (hr)	Mean	SEM	*n*
0.5	1.53	0.49	5
1	3.28	1.06	7
2	2.21	0.47	7
3	0.79	0.10	6
4	0.81	0.19	6
6	0.52	0.11	6
24	0.45	0.12	6

Table 3. Experiment 2B data for unconjugated serum ^{3}H-BPA in adult female CD-1 mice at 24 hr after a single oral administration of varying doses of ^{3}H-BPA. Values at each dose were from different animals.

Dose (µg/kg)	Mean	SEM	*n*
2	0.0058	0.00	7
20	0.03	0.01	4
400	0.58	0.14	6
100000	167.96	30.04	5

Table 4. Experiment 2C data for unconjugated and conjugated serum BPA in adult female CD-1 mice over the 24 hr after a single oral administration of 100,000 µg/kg BPA. Each value is derived from one pool of blood from 4 mice.

Time (hr)	Unconjugated BPA, ng/mL	Conjugated BPA, ng/mL
0	0.000	0.904
0.5	429.63	20414.069
1	949.136	114151.862
2	323.667	40844.218
3	201.77	30383.479
4	109.013	14673.340
6	125.142	17341.327
24	7.704	601.701

Table 5. Experiment 3 data for conjugated serum dBPA in women over the 24 hr after a single oral administration of 69.3 µg/kg BPA. Data were obtained from Volkel et al. (2002) using GraphClick software and converted from nM to ng/mL using standard methods.

	dBPA, ng/mL		dBPA, nM		
Time (hr)	Mean	SEM	Mean	SEM	*n*
0	0	0	0		
4	24.050	9.523	105.485	72.344	3
8	14.444	4.637	63.352	35.226	3
12	5.291	0.426	23.207	3.240	3
16	3.174	2.330	13.921	17.702	3
24	0.606	0.304	2.656	2.307	3

PHARMACOKINETIC AND STATISTICAL ANALYSES USING WinNonlin AND NONMEM SOFTWARE

1. Non-compartmental analysis (experiment 1 with rhesus monkeys and experiment 2A with mice)

Serum concentration-time profiles were analyzed with a Non-Compartmental Analysis (NCA) using WinNonlin (WinNonlin® professional version 5.3 Pharsight Corporation, Cary, NC, USA). Area under the curve (AUC) up to the last quantifiable serum concentration, i.e. $AUC_{(0\text{-}Clast)}$, was calculated by using the linear trapezoidal rule. Extrapolation to infinity to obtain $AUC_{(0\text{-}infinity)}$ was calculated by dividing the last observed quantifiable serum concentration by the slope of the terminal phase as estimated by linear regression using the best fit option of WinNonlin. Mean Residence Time (MRT), which refers to the average total time BPA molecules of a given BPA dose spend in the body, was obtained with and without extrapolation to infinity by using statistical moments (Gibaldi and Perrier 1982). MRT can be viewed as the arithmetic mean of times that each BPA molecule spends in the body, and it is a metric of persistency of BPA in the body because it is a stochastic view of BPA pharmacokinetics (PK) in the body.

The apparent oral clearance (Cl/F) was obtained by dividing the administered BPA dose by the corresponding $AUC_{(0\text{-}infinity)}$ or $AUC_{(0\text{-}Clast)}$, C_{last} being the last quantifiable serum BPA concentration. For mice administered 400 µg/kg BPA, there was a single point per mouse and 6 or 7 mice per sampling time, and the sparse data option of WinNonlin was used, allowing computation of the different standard errors (SE) associated with estimated parameters. Definitions of the different computed parameters are given in Table 6.

Table 6. Definition of the pharmacokinetic parameters computed using a non-compartmental analysis for rhesus monkeys and mice.

PK Parameter	Definition
AUC_%Extrap_obs	Percentage of AUCINF_obs that is due to extrapolation from Tlast to infinity; extrapolation done with lambda_z; AUC: Area under the curve
AUCINF_obs	AUC from time of dosing (0) to infinity; extrapolation with the last quantifiable (i.e. above LOQ) concentration divided by the terminal slope (lambda_z)
AUClast	AUC from time of dosing (0) to the time of the last quantifiable concentration
Cl_F_obs	Apparent total serum clearance for extravascular administration (or oral clearance) calculated from AUCINF_obs
Cl_F_last	Apparent total serum clearance for extravascular administration (or oral clearance) calculated from AUClast
Clast	Concentration observed at Tlast
Cmax	Maximal serum BPA concentration
HL_Lambda_z	Terminal half-life (ln(2)/terminal slope); best fit option of WinNonlin
MRTINF_obs	Mean Residence Time (MRT) extrapolated to infinity using the last quantifiable serum concentration for extrapolation
MRTlast	Mean Residence Time (MRT) from time of dosing to the last quantifiable serum BPA concentration
Tlast	Time of last quantifiable serum concentration
Tmax	Time of maximal serum BPA concentration

Additional output	Definition
Corr_XY	Correlation between time (X) and log concentration (Y) for the points used in estimation of the terminal slope (lambda_z)
SE_AUClast	Standard error (SE) associated with AUClast estimate for sparse data analysis in WinNonlin (mouse data)
SE_Cmax	Standard error (SE) associated with Cmax estimate for sparse data analysis in WinNonlin (mouse data)

Results of the non-compartmental data analysis for the monkey are given in Table 7.

Table 7. Pharmacokinetic parameters for unconjugated BPA obtained by a non-compartmental data analysis in rhesus monkey; BPA dose of 400 µg/kg by oral route.

Parameter	Days	Units	Mean	SE	Min	Median	Max
AUC_%Extrap_obs	Day1	%	16.7	4.8	5.1	10.2	48.4
AUC_%Extrap_obs	Day7	%	8.2	1.2	3.7	7.9	14.1
AUCINF_obs	Day1	hr*ng/mL	13.7	2.6	5.2	10.9	33.9
AUCINF_obs	Day7	hr*ng/mL	10.7	1.1	6.4	9.4	16.4
AUClast	Day1	hr*ng/mL	10.7	2.0	4.6	8.9	26.5
AUClast	Day7	hr*ng/mL	9.5	1.1	6.0	8.2	15.8
Cl_F_obs	Day1	mL/hr/kg	36759	5491	11784	36954	76707
Cl_F_obs	Day7	mL/hr/kg	40879	4330	24457	42719	62424
Clast	Day1	ng/mL	0.66	0.21	0.22	0.42	2.59
Clast	Day7	ng/mL	0.51	0.15	0.20	0.36	1.77
Cmax	Day1	ng/mL	4.29	0.59	1.96	3.97	8.87
Cmax	Day7	ng/mL	4.46	0.51	2.15	4.22	7.94
Corr_XY	Day1		-0.93				
Corr_XY	Day7		-0.95				
HL_Lambda_z	Day1	hr	2.64	0.87	1.00	1.74	10.23
HL_Lambda_z	Day7	hr	1.75	0.32	0.83	1.51	3.57
MRTINF_obs	Day1	hr	4.05	1.31	1.73	2.66	15.47
MRTINF_obs	Day7	hr	2.74	0.45	1.58	2.09	5.46
MRTlast	Day1	hr	2.43	0.67	0.96	1.88	8.93
MRTlast	Day7	hr	1.97	0.27	1.00	1.64	3.55
Tlast	Day1	hr	7.45	1.79	2	8	24
Tlast	Day7	hr	6.6	1.1175	2	6	12
Tmax	Day1	hr	0.91	0.06	0.5	1	1
Tmax	Day7	hr	0.95	0.05	0.5	1	1

Raw data for mouse corresponding to Experiment 2A (BPA dose of 400 µg/kg) are shown in Figure 1 and results of the NCA are given in Table 8.

Table 8. Pharmacokinetic parameters for unconjugated BPA obtained by a non-compartmental data analysis in mice; BPA dose of 400 µg/kg by oral route.

Parameter	Units	Estimate
AUC_%Extrap_obs	%	45.67
AUCINF_obs	hr*ng/mL	29.94
AUClast	hr*ng/mL	16.26
SE_AUClast	hr*ng/mL	1.78
Cl_F_obs	mL/hr/kg	13361
Cl_F_last	mL/hr/kg	24593
Clast	ng/mL	0.403
Cmax	ng/mL	3.28
SE_Cmax	ng/mL	1.06
Corr_XY		-0.862
HL_Lambda_z	hr	23.52
MRTINF_obs	hr	30.9
MRTlast	hr	8.2
Tlast	hr	24
Tmax	hr	1

In Tables 7 and 8, apparent oral clearances (Cl/F) are reported for monkeys and mice, respectively. Cl refers to the systemic clearance after intravenous administration, and F refers to the unknown BPA oral bioavailability (from 0 to 1); F can be estimated from the present data if it is assumed that: (1) the BPA absorption from the gastrointestinal tract is total, (2) BPA is only metabolized by the liver with no renal elimination of unchanged compound, (3) BPA pharmacokinetics is linear, and (4) BPA serum clearance is equal to BPA blood clearance. Under these assumptions, the apparent oral clearance (Cl/F) is an estimate of the BPA intrinsic clearance ($Cl_{intrinsic}$) (see Gibaldi and Perrier, page 332-334 for explanation), and then the overall bioavailability of BPA after an oral BPA administration can be estimated by the following relationship (Equation 1):

$$F = \frac{Qh}{Qh + (Dose / AUC_{oral})} = \frac{Qh}{Qh + Cl_{intrinsic}} \qquad \text{Eq 1}$$

In Equation 1, Dose is the administered BPA dose (400 µg/kg), AUC_{oral} is the estimated $AUC_{(0\text{-infinity})}$ as reported in Table 7 for monkey or the estimated $AUC_{(0\text{-Clast})}$ as reported in Table 8 for mouse, and Qh is the hepatic blood flow. In the present experiment, the estimated oral clearance in rhesus monkeys was 36759 mL/kg/hr (first day) and 40879 mL/kg/hr (seventh day) (Table 7). Using a mean value for the BPA oral clearance of 647 mL/kg/min, a hepatic blood flow of 35 mL/kg/min in rhesus monkeys and solving Equation 1 give an estimate of F of 5.13%.

The hepatic BPA extraction ratio (Eh) of BPA in rhesus monkeys is given by:
$E_h = 1 - F = 1 - 0.05 = 0.95$

This means that the hepatic first pass-effect (95%) of BPA in rhesus monkeys is large but not total.

Using the same approach and the same hypotheses, and considering that the hepatic blood flow in mice is about 100 mL/kg/min, the intrinsic clearance of BPA in mice was estimated to be 24593 mL/kg/hr (= 410 mL/kg/min); F was estimated to be 19.6%, and the hepatic extraction ratio to be about 0.80, indicating that internal exposure to parent BPA by the oral route is greater in mice than in rhesus monkeys (all other things being equal), because the apparent hepatic first-pass effect is only about 80% in mice.

2. Compartmental analysis (experiment 1 in rhesus monkeys)

A so-called population pharmacokinetic analysis was performed on BPA serum concentration data obtained in female rhesus monkeys on day 1 and day 7 after administration of 400 µg/kg/day BPA by the oral route. The objective of the analysis was to properly analyze the data in monkeys, which contained a number of measurements of BPA concentrations below the LOQ of 0.2 ng/mL (38%).

The software used for the analysis was NONMEM software version VI (GloboMax, ICON Development Solutions, Ellicott City, MD), and the estimation method was the FOCE-I method (first-order conditional estimation with interaction). A total of 157 observations were included in the analysis, corresponding to all quantifiable (N = 97) and non-quantifiable (N = 60) concentrations obtained in Experiment 1, apart from the samples taken prior any BPA administration. For the non-quantifiable concentrations, the information that these concentrations were below the LOQ was taken into account when computing the likelihood, according to a previously described method (Method M3 in Ahn et al. 2008).

The nonlinear mixed effects model shown in Equation 2 was used for the analysis.

$$Y_{ij} = f(D,\psi_i,t_{ij}) + g(D,\psi_i,t_{ij},\sigma)\varepsilon_{ij} \qquad \text{with} \qquad \varepsilon_{ij} \overset{iid}{\sim} N(0,1) \qquad \text{Eq 2}$$

In Equation 2, Y_{ij} is the observation in the i^{th} monkey *(*i = 1…11*)* at time t_{ij} (j = 1…n_i), and n_i being the number of observations per animal; D refers to the dose(s) administered, ψ_i is the vector of individual pharmacokinetic parameters in the i^{th} monkey, σ is a vector of unknown real constants, and ε_{ij} is a random variable accounting for the residual error (analytical error, model misspecification); g denotes the function depending on D, ψ_i, t_{ij} and σ that codes for the residual error model; and f denotes the function depending on D, ψ_i and t_{ij} that codes for the structural pharmacokinetic model after single or repeated doses. In the case of a two-compartment model with first-order absorption and for a single dose administration, f is expressed as follows in Equation 3.

$$f(t) = \frac{D}{(V_c / F)}\left(Ae^{-\alpha t} + Be^{-\beta t} - (A+B)e^{-Kat}\right) \quad \text{Eq 3}$$

In Equation 3, α (1/hr) is the rate constant of the initial phase, β (1/hr) is the rate constant of the terminal phase, K_a (1/hr) is the absorption rate constant, V_c/F (L/kg) is the apparent central volume of distribution, *A* and *B* (no unit) are macroconstants, and *t* is the time after dose (hr).

Model parameterisation was in macroconstants in order to estimate directly the rate constants of the different phases. For a two-compartment model with first-order absorption, the corresponding parameterisation in NONMEM is in K_a, α, β, A/B and V_c (ADVAN4 TRANS5) so that $\psi_i^t = (Ka_i, \alpha_i, \beta_i, (A/B)_i, (Vc/F)_i)$.

At the population level, it was assumed that the individual pharmacokinetic parameters ψ_i were log-normally distributed, as shown in Equation 4.

$$\log(\psi_i) = \log(\theta) + \eta_i \qquad \text{with} \qquad \eta_i \overset{iid}{\sim} N(0,\Omega) \quad \text{Eq 4}$$

In Equation 4, θ is an unknown vector of fixed parameters (or fixed effects), η_i is the vector of real random effects associated with subject i and accounting for inter-individual variability and Ω is a variance-covariance matrix; η_i and ε_{ij} are assumed independent.

For the sake of simplicity and given the data, we assumed that Ω was diagonal.

Model selection was based on the objective function (defined as minus twice the log-likelihood up to an additive constant), basic diagnostic plots and inspection of standard errors for model parameter estimates. Differences in objective function between nested models were tested by using the likelihood ratio test. Different models were investigated for the residual error, such as the proportional error model: ($g(D,\psi_i,t_{ij},\sigma) = \sigma \times f(D,\psi_i,t_{ij})$), and the combined error model: ($g(D,\psi_i,t_{ij},\sigma) = \sigma_1 \times f(D,\psi_i,t_{ij}) + \sigma_2$).

The adequacy of the final selected model was evaluated through visual predictive checks. Visual predictive checks are a Monte Carlo simulation based method that compares graph observations with model predictions as a function of time. Specifically, 1000 replicates of the study design were simulated with the final model (1000×11 simulated monkeys in total). The distribution of model predicted concentrations was summarized at each time point by the 50th percentile (median) as well as the 5th and 95th percentiles delineating the 90% prediction interval. These percentiles were then plotted against time and superimposed with observations. Model simulations were also used to derive the 90th and 95th percentiles of the terminal half-life (calculated as log(2)/β) together with the corresponding 95% confidence intervals (denoted $CI_{95\%}$). All graphs were created by R software version 2.7.2.

Based on all quantifiable and non-quantifiable unconjugated dBPA serum concentration data, the final selected model was a two-compartment model with first-order absorption, with

inter-individual variability on the apparent central volume of distribution (V_c/F) and on the terminal phase rate constant (β). Inter-individual variability on K_a, α and A/B could not be properly estimated given the data. A combined error model (with additive and proportional components) was selected for the residual error.

This final model was judged to adequately describe BPA concentrations in rhesus monkeys based on the visual predictive checks (Figure 2); indeed, observations lie mainly within the 90% prediction interval of the model predictions at each time point. Model parameter estimates are displayed in Table 9. Estimations of the geometric means of α and β were 1.58 and 0.298 1/hr, respectively. Mean half-lives were calculated from these population estimates, giving 0.44 hr (26 min) for the initial phase and 2.32 hr for the terminal phase. Relative standard errors of mean half-lives were derived from the relative standard errors of α and β population estimates and were actually the same (17 and 27%, respectively).

Given the large inter-individual variability estimated on β, a large inter-individual variability is predicted by the model on the terminal half-life. According to the model, 10% of the subjects are expected to have a terminal half-life above 6.3 hr ($CI_{95\%}$ of [2.8; 10]), and 5% of the subjects to have a terminal half-life above 8.2 hr ($CI_{95\%}$ of [3.4; 14]). It is noteworthy, however, that estimation of inter-individual variability was based on only 11 monkeys. Overall, the relative short half-lives regarding the 24 hr dosage interval explain the lack of accumulation for BPA following repeated daily administrations.

Please note that models with inter-occasion variability were tested during the model building, since BPA serum concentrations were measured on two different occasions (day 1 and day 7). Here, inter-occasion variability refers to the difference in individual pharmacokinetic parameters between day 1 and day 7 in a given monkey. This difference is regarded as random and was modelled in terms of random variables κ. Inter-occasion variability, however, could not be properly estimated from the data and was not included in the final model.

Table 9. Parameter estimates of the population pharmacokinetic model developed for unconjugated dBPA in the 11 adult female rhesus monkeys. Relative standard errors (calculated as SE/estimate×100 and denoted RSE) are provided in parentheses. As variance estimates refer to the variance of log-transformed individual pharmacokinetic parameters, the coefficients of variation of untransformed individual pharmacokinetic parameters are also displayed (in square brackets).

Pharmacokinetic parameter	**Geometric mean estimate**	**Variance estimate [CV%]**
Absorption rate constant K_a (1/hr)	1.46 (17)	- [a]
Initial phase rate constant α (1/hr)	1.58 (17)	- [a]
Terminal phase rate constant β (1/hr)	0.298 (27)	0.597 (76) [90%]
Ratio of macroconstants *A/B* (no unit)	6.01 (9.7)	- [a]
Apparent central volume *Vc/F* (L/kg)	41.3 (13)	0.0431 (68) [21%]

Residual error model	Estimate
proportional coefficient σ_1 (%)	35.5 (16)
additive coefficient σ_2 (ng/mL)	0.122 (21)

[a] Variances expressing inter-individual variability were not estimated but fixed to zero in the model for K_a, α and *A/B*.

3. Dose proportionality (experiment 2B in mice)

Different statistical analyses were used for the assessment of BPA dose proportionality between BPA doses of 2 to 100,000 µg/kg: (i) dose normalization (scaling) of the BPA serum concentrations by the administered BPA nominal dose (from 2 to 100,000 µg/kg) followed by an analysis of variance (ANOVA) with the dose level as factor; (ii) testing linearity of BPA disposition for the entire BPA dose range (from 2 to 100,000 µg/kg and from 2 to 400 µg/kg).

The linearity of BPA disposition over the entire BPA dose range (see Figure 3 in the published article) was first tested with a power model of the form shown in Equation 5

$$Y = \alpha X^{\beta} EXP(\varepsilon) \qquad \text{Eq 5}$$

In Equation 5, β is the power term, *Y* represents the dependent variable (here BPA concentration at 24 hr post BPA administration), *X* represents the dose, and **ε** is a residual term. Following a logarithmic transformation of both sides, the relationship between log(concentration) and log(dose) becomes a linear relationship, to which a linear regression approach can be applied as shown in Equation 6.

$$\log(Y) = \log(\alpha) + \beta \log(X) + \varepsilon$$ Eq 6

Assuming that the underlying relationship between log(concentration) and log(dose) is linear, a value of 1 for β indicates perfect dose proportionality. Therefore, the estimate of β together with a suitable Confidence Interval (CI) can be used to quantify dose proportionality. The advantage of this model is that it generally stabilizes variance. To interpret the slope, an equivalence approach was used as explained by Smith et al. (2000) to accept or reject the hypothesis that the slope is close to 1. The *a priori* acceptable CI for the slope is given by the following relationship shown in Equation 7.

$$1 - \frac{\log(0.8)}{\log(dose\_ratio)} < slope < 1 + \frac{\log(1.25)}{\log(dose\_ratio)}$$ Eq 7

Here 0.8 and 1.25 are the critical values suggested by regulatory authorities for any bioequivalence problem after a data log transformation. Using this equivalence approach, dose proportionality was not demonstrated either for the 2 to 100,000 or for the 2 to 400µg/kg doses. Consequently, dose proportionality and linearity were tested without fixing an *a priori* equivalence interval using weighted linear regression (Weight= $1/Y^2$) between the nominal dose (from 2 to 400 µg/kg) and observed plasma concentrations. First a polynomial equation including a quadratic term (see Equation 8) was used to assess a possible lack of fit regarding the linear model corresponding to a simple straight line.

$$Y = \alpha + \beta_1 X + \beta_2 X^2 + \varepsilon$$ Eq 8

Here the hypothesis is whether or not β_2 equals zero; if β_2 is not significantly different from 0, the simple linear weighted regression (Weight=$1/Y^2$) is accepted, and then Equation 9 is used to test linearity/ proportionality.

$$Y = \alpha + \beta X + \varepsilon$$ Eq 9

Here the hypothesis that α=0 is tested; if α=0, then the BPA disposition is said to be linear, and serum concentrations increase with the administered dose, with proportionality coefficient β.

All regressions were performed using WinNonlin Professional software (WinNonlin, version 5.0.1, Pharsight Corporation, Mountain View, CA, U.S.A.). Goodness of fit was determined by visual inspection of the fitted curve and of the residuals scatter plot.
The results were as follows: the mean serum BPA concentrations scaled by the administered dose were rather similar across the tested doses, with no trend. The P value associated with the one way ANOVA was P=0.5059, indicating that the null hypothesis (dose proportionality) could not be rejected. This conclusion is not equivalent to the conclusion saying explicitly that there is evidence of dose BPA proportionality, and linearity and proportionality were tested using regression models. Data for doses ranging from 2.3 to 98447 µg/kg that were analyzed after a log-log transformation indicated a good fit. Plot of the fitted curve is given by Figure 3; plot of residuals is given in Figure 4. Inspection of these

figures indicates that the log-log transformation stabilized variance (i.e. homoscedasticity is obtained).

Thus, the results of the regression were considered with an estimated slope of 0.979942. The univariate 95% CI for the slope was 0.9165-1.0433, and the shortest 90% CI was 0.9275-1.032; this is the classical shortest interval computed for a bioequivalence problem. The BPA dose ratio tested (higher vs. lower tested dose) was 98,447/2.3=42,803. Thus, the *a priori* confidence interval for this BPA dose ratio was 0.9790-1.0209 (see Equation 7); it can be concluded that both the 95% and the 90% CI for the slope were not totally included within this *a priori* regulatory recommended CI, and that BPA dose proportionality cannot be proved for this full range of BPA doses; as explained by Smith et al. (2000), as the dose ratio increases, the critical region for the slope narrows. It is intuitive that the criterion for proportionality should be more stringent for a large dose range than that for a narrow range. Data for doses ranging from 2.3 to 396.9 µg/kg were also analyzed after a log-log transformation, and using the shortest 90% CI, it was impossible to make a conclusion about BPA dose proportionality.

From the weighted linear regression approach, however, there was no evidence of a lack of fit (β_2 not significantly different from 0 in Equation 8); then using Equation 9, it was shown that the intercept (α in Equation 8) was not significantly different from 0 (Figures 5 and 6), and the hypothesis of dose linearity was accepted for the BPA dose range from 2 to 400 µg/kg.

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C. FIGURES

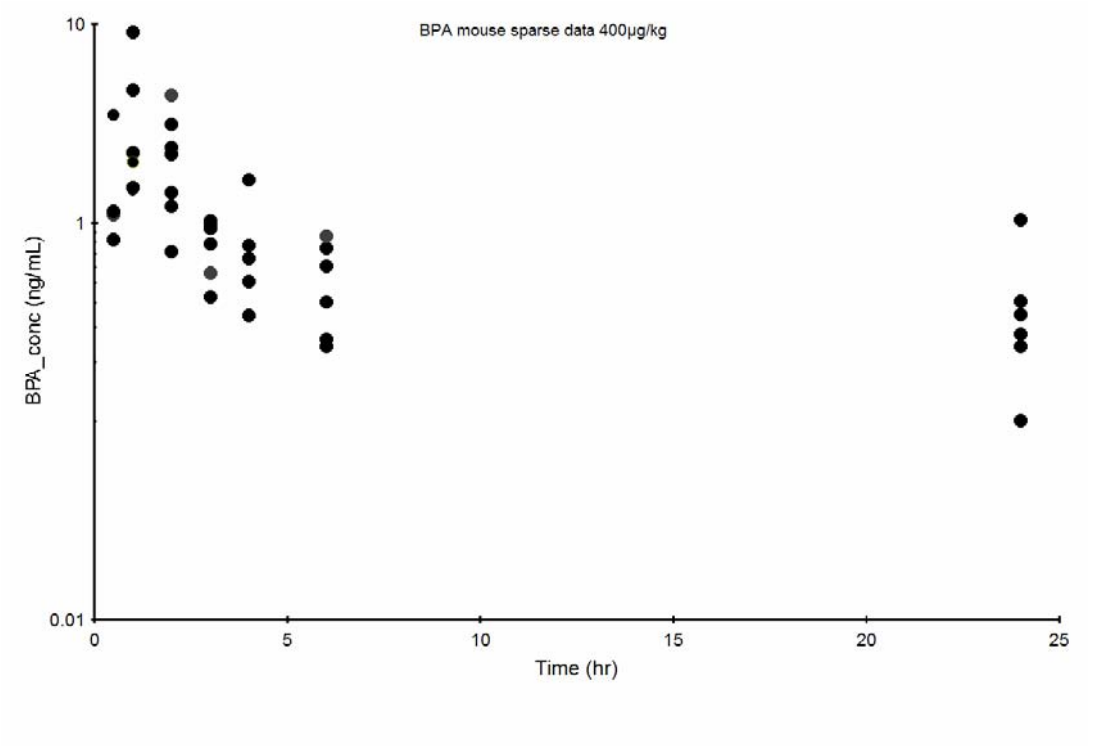

Figure 1: Semi-log plot of unconjugated BPA serum concentrations in mice after an oral BPA administration at 400 µg/kg (1 point per mouse, 6 – 7 mice per sampling time).

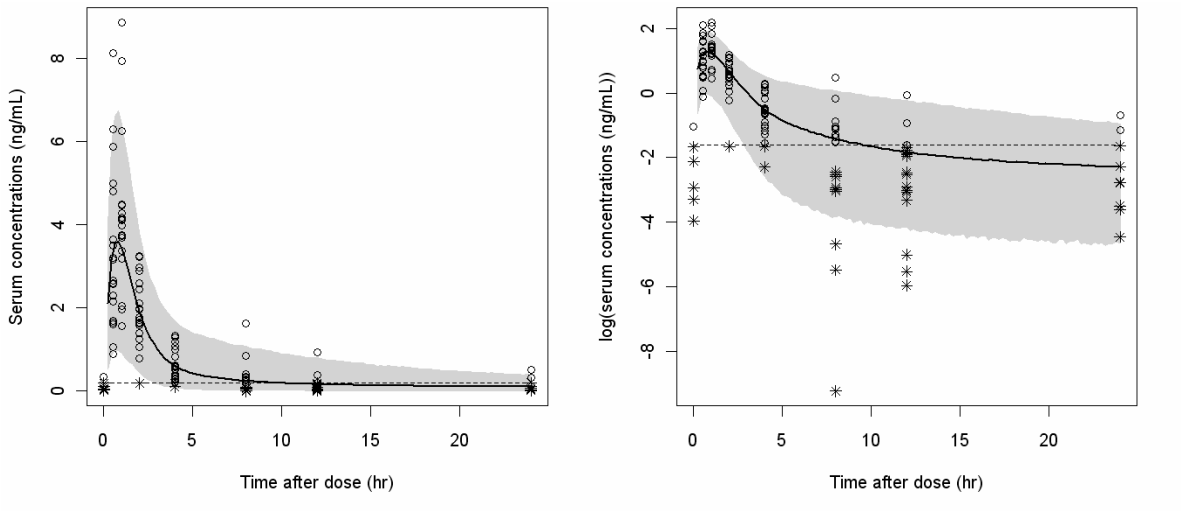

Figure 2. Visual predictive checks comparing unconjugated dBPA serum concentrations observed in the 11 adult female rhesus monkeys with their predictions according to the population pharmacokinetic model (left: Cartesian scale; right: semi-logarithmic scale). Model predictions were generated by Monte Carlo simulation using the final model parameter estimates and the study design. They are summarized at each time point by their median (bold line) and their 90% prediction interval (grey area). Observations above the LOQ (dashed line) are represented by dots, while observations below the LOQ are represented by stars. Note that only strictly positive values could be plotted on the graph with the semi-logarithmic scale. Since no differences between day 1 and day 7 were found, all data were plotted on the same graph as a function of the time after dose.

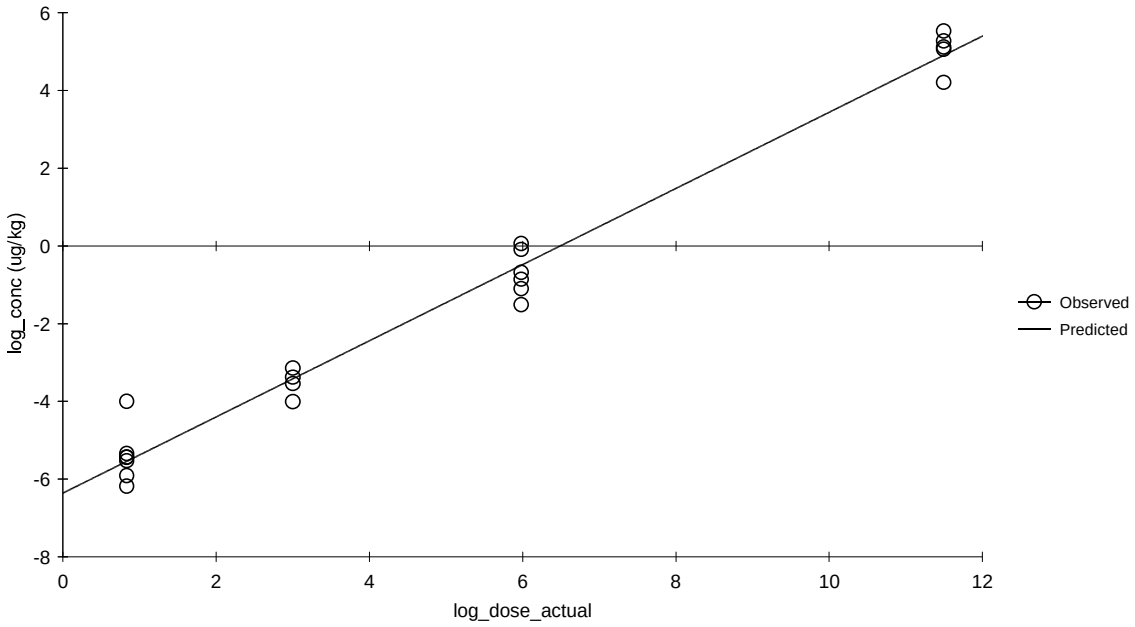

Figure 3: Observed Y and Predicted Y for the power (linear log-log) model, with data corresponding to doses ranging from 2.3 to 98,447 µg/kg (log-log scale); visual inspection of Figure 3 gives good apparent fit.

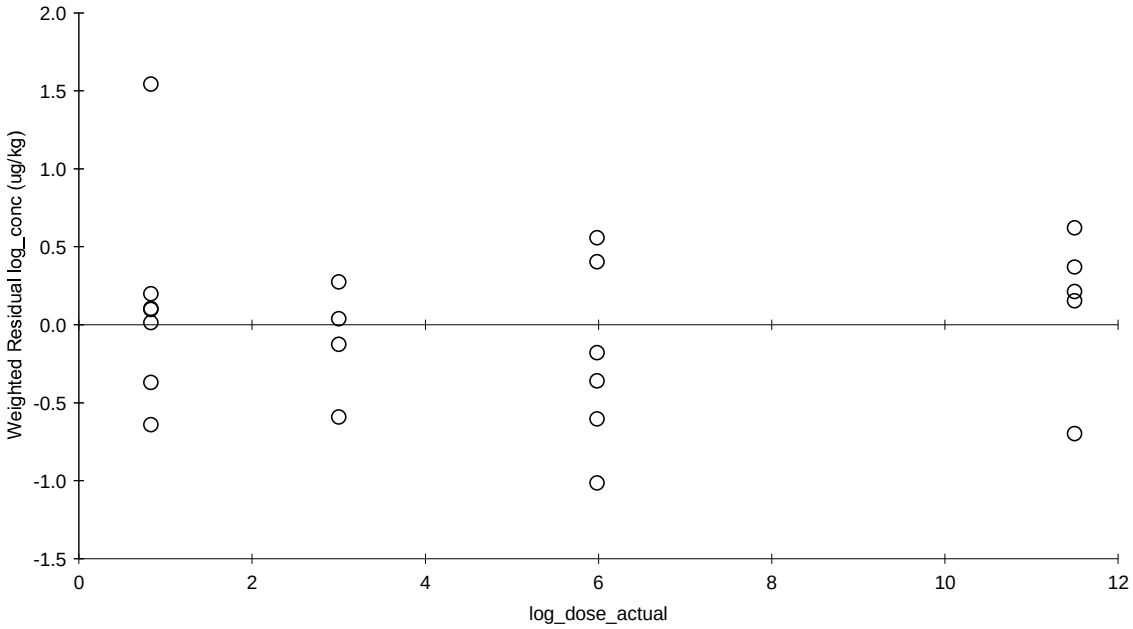

Figure 4: X vs. weighted residual Y for a log-log linear power model with data corresponding to doses ranging from 2.3 to 98,447 µg/kg; inspection of Figure 4 indicates appropriate scatter of residuals (no bias, homoscedasticity).

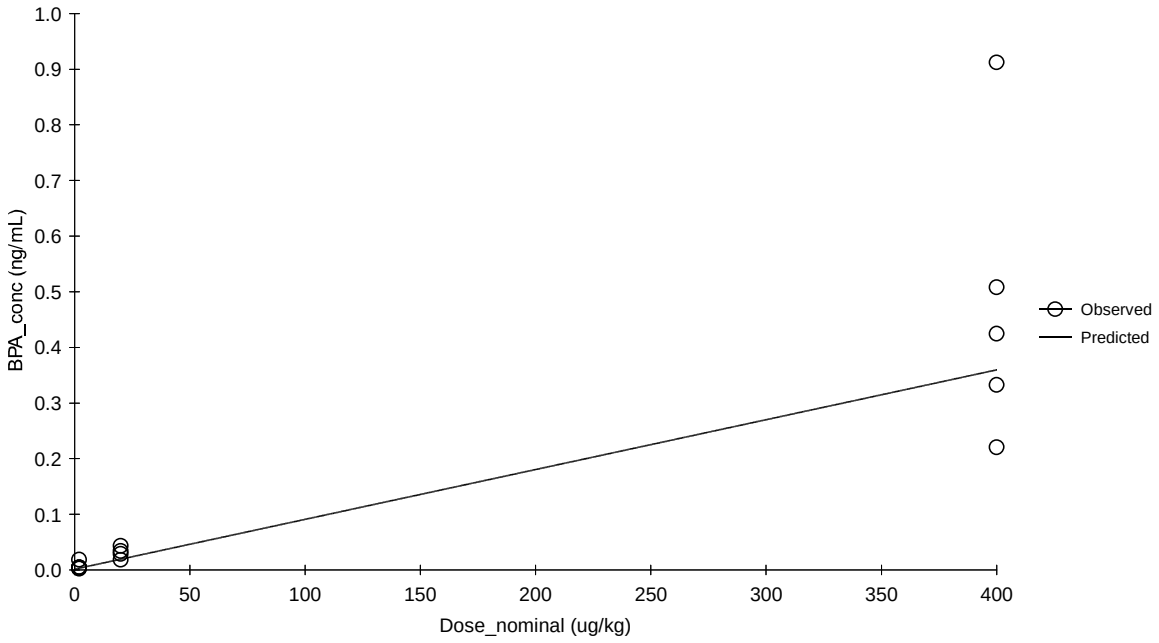

Figure 5: Observed Y and Predicted Y for the simple weighted linear model with data corresponding to dose ranging from 2.3 to 400 µg/kg; visual inspection of Figure 5 indicates good fit. Data were analyzed by a simple weighted ($1/Y^2$) linear model with data corresponding to doses ranging from 2.3 to 400 µg/kg.

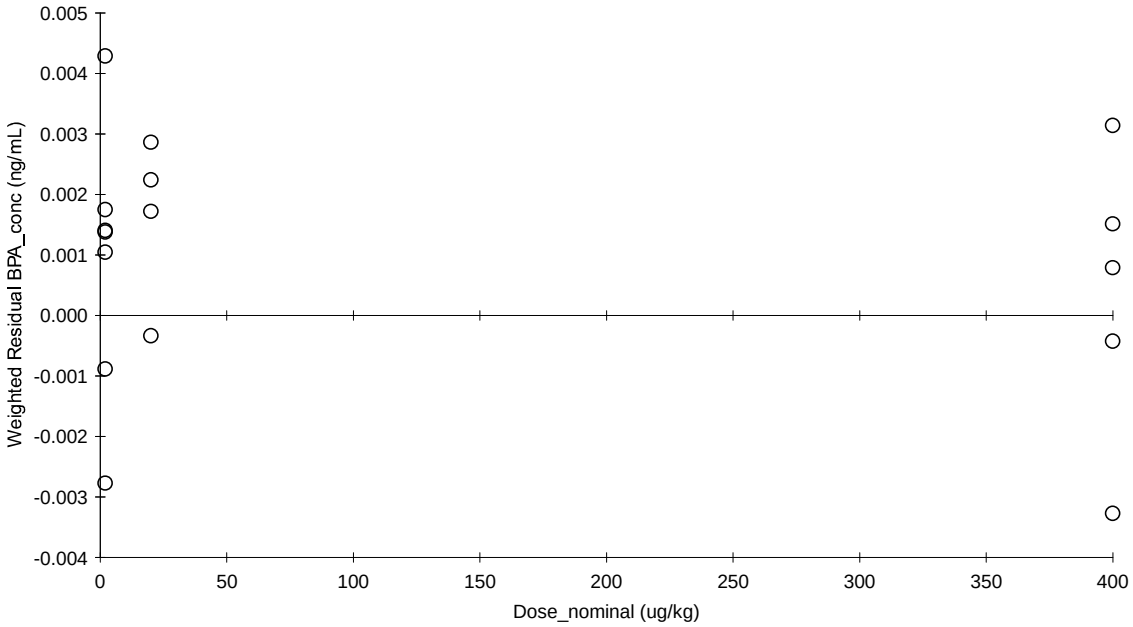

Figure 6: X vs. Weighted Residual Y for a simple linear model with data corresponding to BPA doses ranging from 2.3 to 400 µg/kg; inspection of Figure 6 suggests homoscedasticity and lack of misfit. Data analyzed by a simple weighted ($1/Y^2$) linear model with data corresponding to doses ranging from 2.3 to 400 µg/kg.